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(54) Interleukin therapy

(57) Liposomes containing interleukin are advantageous sustained release formulations for interleukin therapy.

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SPECIFICATION

Improvements in or relating to interleukin therapy

This invention relates to interleukin therapy.

Interleukin-2 (hereinafter IL-2) is a naturally occurring protein factor first discovered in 1976. It is produced from T-cells activated by an antigen or lectin and is a factor essential for the proliferation of T-cells. IL-2's of various structures have been isolated from a number of animal species, e.g. mouse, primate such as the gibbon, ape, and human. Human and other IL-2's have been purified from various sources such as peripheral blood lymphocytes, tonsilar lymphocytes, spleen lymphocytes, T-cell leukemia and T-cell hybridoma cultures.

As indicated above IL-2 is a factor essential for the proliferation of T-cells, themselves intimately involved in the body's immune response mechanisms. IL-2 is anticipated to have therapeutic potential of immense proportions. In particular, it may have value in the therapy of tumours since it leads to proliferation of antigen specific T-cells e.g. against tumour antigens and these T-cells can inhibit tumour growth. It is also known that IL-2 induces production of gamma-interferon and activates natural killer cells. It is also expected that IL-2 will have a variety of applications against immunological disorders, such as neoplatic diseases, bacterial or viral infections, immune deficient diseases, auto-immune diseases etc (see B.Papermaster et al, Adv. Immunopharm. (1980) 507).

As with other naturally occurring products such as the interferons, before the advent of genetic engineering, IL-2 was available in small quantities only.

However, following methodology similar to that previously published for other proteins (e.g. gamma-interferon) the structure determination and expression of a cloned gene for human IL-2 was effected (see S.Taniguchi et al, Nature (1983) *302*, p.305-310 and European Patent Publication 91539 the contents of both which are incorporated by reference. This particular IL-2 is referred to hereinafter as known IL-2.

On the basis of the nucleotide coding sequence the amino acid sequence of the human IL-2 polypeptide, namely that comprising the amino acids numbered 1 to 153 in Fig.3a of the above Nature article, was deduced. It was postulated, however, that the first 20 animo acids of this sequence could be cleaved on transmembrane transport. The mature human IL-2 would then consist of 133 amino acids beginning with the A1a at position 21.

This structure of human IL-2 has since been confirmed in our and other laboratories.

It is also possible by using recombinant DNA techniques to produce IL-2's of different structures. For example, modifications of the human IL-2 polypeptide having one or more amino acids absent or replaced by other amino acids, e.g. as described in the above mentioned European Patent Publication, in particular pages 23 to 25 thereof, may be produced by correspondingly modifying the human IL-2 gene. For example, if desired Cysteine residues may be replaced by other amino acids, e.g. serine, as described in Cetus European Patent Publication 109748 and Belgium Patent 898016, e.g. IL-2-serine- 125, the contents of which are hereby incorporated by reference.

Further examples of such interleukins are disclosed in PCT patent publication WO 85/30817, the contents of which are incorporated herein by reference. These include IL-2 Gln-26, IL-2 Phe-121, and IL-2 Stop 121.

Furthermore, IL-2's from different sources such as the gibbon ape may be produced by recombinant DNA techniques and modification of these may also be produced in similar manner. Furthermore, IL-2's may, as indicated, be isolated (albeit in relatively small quantities) from natural sources and may differ from the products produced by recombinant DNA technology in for example glycosylation. Moreover, allele variantes may be produced.

IL-2's may also be produced by cultivating human cells, e.g. in the presence of an inducer. Examples are IL- 2 A-1, IL-2 A-2, IL-2 B-1, IL-2 B-2 as defined in Danish Patent Application No. 3317/84 and European Patent Publication No. 132359, the contents of which are incorporated herein by reference.

By "interleukin" as used hereinafter is meant any polypeptide, including but not limited to those described above, whether isolated from natural sources or produced by a synthetic or biosynthetic method and which has IL-2 activity. In accordance with the present invention, the preferred interleukin is human IL-2 or a modification thereof, preferably as produced by recombinant DNA methods. Trials so far carried out with interleukin have been by injection, e.g. intravenously. These have shown for example that human IL-2 has a very short half-life of under 5 minutes. There is clearly a need for a sustained release parenteral form of interleukin providing a sufficiently long duration of action, however, up to now little has been published n specific galenic formulations on interl ukins.

Furthermore, trials carried out in our laboratories hav shown that interleukin may be incorporated into lipsomal forms, which on administration, .g. intraven us administration, still show IL-2 activity. Furthermore, the lipsomes f the present invention have a prolonged half-life, are passively targetted into the spleen, lung, bone marrow or lymph nodes.

The present inv nti n accordingly provides interleukin - containing liposom s.

Liposomes acc rding to the invention may be prepared by encapsulating an interleukin in a liposome forming material.

Liposomes are completely closed bilayer membranes containing an aqueous phase with the interleukin. The interleukin may be in the aqueous phase and/or in the membrane. They may be in the form of a unilamellar vesicle or a oligo- or multi-lamellar vesicle in the form of concentric membrane bilayers each separated from one another by a layer of water.

They may be made by variety of different methods - see F.Szoka and D.Papahadjopoulos, "Liposomes and their uses in biology and medicine" A.N.Y.Acad.Sci. *308*, 1-462, (1978); R.L.Juliano & D.Layton, "Liposomes as a drug delivery system" in Drug delivery systems p.189-236, Oxford University Press, Inc., New York, 1980, whose disclosure is incorporated by reference herein, but the method of the invention is preferred as it provide liposomes which have particularly interesting properties. Thus the liposomes produced by this method are especially stable, e.g. against leakage of interleukin and the method of the invention is suitable for commercial production.

The liposomes may be made from a variety of lipids capable of forming vesicle walls. Preferred lipids are phospholipids such as natural lecithin (e.g. egg or soyabean lecithin), synthetic lecithins, kephalines and sphingomyelins. Alternatively, other phosphatidylcholines, phosphatidic acids, lysophosphatidylcholines, sphingolipids, phosphatidylglycerol, cardiolipins, glycolipids, gangliosides and cerebrosides may be used.

Examples of lecithins includes EPIKURONS available from Lucas Meyer, Hamburg 28, W.Germany. One example contains the following phospholipid content: Phosphatidyl choline 44-7%, Phosphatidylethanolamine 22-25%, Phosphatidyl inosit 0-2%, fatty acid content: saturated-palmitic acid 9-11%, stearic acid 2-4%, unsaturated: linoleic acid 63-67%, linolenic acid 5-8%, oleic acid 14-18%, known as EPIKURON 145 V. An alternative example contains greater than 95% phoslatidyl choline e.g. 95-98% and predominantly unsaturated acids, e.g. oleic acid 10- 12%, linoleic acid 62-65%, linoleic acid 5-6%, as well as saturated acids, e.g. palmitic acid 15-17%, stearic acid 3-4%, such as EPIKURON 200.

It is preferred to have a high content of unsaturated acids. If desired a lecithin with a high content of saturated acids, e.g. stearic acid, and phosphatidyl choline 95% or greater, e.g. SOJA PHOSPHITID NC 95 or pure equivalents suitable for pharmaceutical use, available e.g. from NATTERMANN CHEMIE GmbH, KOELN, W.Germany.

Synthetic phospholipids may contain altered aliphatic portions such as hydroxyl groups, branched carbon chains, cyclo-derivates, aromatic derivatives, ethers; amides, polyunsaturated derivatives, halogenated derivatives or altered hydrophilic portions containing carbohydrate, glycol, phosphate, phosphonate, quaternary amine, sulfate, sulfonate, carboxy,amine, sulfhydryl and imidazole groups, e.g. as in dimyristoyl-, dipalmitoyl- or distearoyl-phosphatocholine derivatives.

If desired in the final liposome the bilayer may contain, e.g. up to 50 (mole) percent of, other lipids, e.g. steroids such as cholesterol.

Preferably in the final liposome a steroid such as cholesterol is present. Suitable weight ratios of lipid to steroid may be from 6:1 to 1:1.

If desired the bilayer may comprise a lecithin, kephaline or sphingomyelin containing up to 10 (mole) percent of an additive to increase incorporation, e.g. anionic compounds such as acids, e.g. dicetyl phsophate, phosphatidic acid, sodium taurocholate, phosphatidyl serine (Merck) or cationic compounds e.g. amines such as stearylamine. It is preferred to use phosphatidylserine (obtainable e.g. from Merck, W.Germany).

When the lipid contains extra excipients it may be preferred to evaporate a solution of the lipid and excipients e.g. in methylene chloride in a reaction vessel to form a lipi form on the vessel surface before the lipid is mixed with the interleukin.

The liposomes may be produced by well-known techniques,e.g. by irradiation e.g. ultrasonic sonification, using mild conditions to prevent breakdown of the interleukin. Homogenous mixtures of lipids may be produced by forming a solution of lipid, e.g. lecithin and cholesterol in a suitable organic solvent, e.g. chloroform. The solution in a reaction vessel is then evaporated to produce a lipid film.

The liposomes may be isolated and sterilized in conventional manner.

In the resultant liposomes according to the invention the concentration of interleukin is preferably from about 5 to about 500 microgram/millilitre of aqueous phase, preferably 20 to 200 microgram/millilitre.

The lipid concentration is preferably from about 1 to about 200 mg/ml of aqueous phase, especially 10 to 100 mg/ml.

The average diameter of the liposomes for further use is preferably from about 25 nanometres to 20 microns, preferably from 100 to 500 nanometres.

The final liposomes are preferably stored at low temperatures e.g. −20°C t + 5°C.

In order to improve their storage the liposomes according to the invention may be lyophilized to a dry powd r, e.g. by fr eze-drying using for example mannitol, sucrose, polyvinylpyrrolidone or gelatin as carri r material. This may be effected under the same conditions for freeze-drying indicated below with respect to step Ab). Befor use they may b reconstituted by the addition of sterile water. They may then be mixed with syst ms appropriate for, for example intraven us injection or infusion.

The purity of the liposomes may be determin d by conventional analytical techniques.

An anti-oxidant may if desired by present up to e.g. 1% preferably 0.1% of the lipid, in the resultant liposomes. Prefered anti-oxidants include Vitamin E (tocopherol acetate), Vitamin C palmitate and BHT (butylated hydroxytoluene). Other stabilizers for the interleukin may be present, e.g. sugar, e.g. mannitol, arabinose, sorbitol, or sucrose, albumins e.g. human serine albumin etc. These may be incorporated into the buffer before liposome formation. Preferably they are present in up to 20% by mole weight of the lipid in the resultant liposome.

A preferred process for liposome formation is the following process a) which comprises

Aa) forming a solution of an interleukin and a lipid in an organic solvent,

Ab) removing the solvent from the solution to give a residue,

Ac) suspending the residue in a solution of a buffer,

Ad) subjecting the suspension to agitation and homogenization until liposomes are produced, and

Ae) isolating the liposomes.

In step Aa) the term "solution" covers "pseudo" solutions like emulsions, However, it is preferred to produce a uniform, clear solution. Any solvent system may be used which will dissolve or solubilize the interleukin and the lipid. The system may be a single solvent or a mixture of solvents. It may contain up to e.g. 15% water. If desired a surfactant may be present. The solvent system may comprise any appropriate organic solvent which can be removed from the lipid by evaporation. A wide variety of ethers, such as diethyl ether, and diisopropyl ether, ester such as ethyl acetate, alcohols such as methanol and tert-butanol, and halogenated hydrocarbons such as methyl chloride and chloroform may be used. If desired acetic acid may be present. It is preferred to use diethylether, methylene chloride or tert-butanol.

We have found that tert-butanol is preferred when high vacuum is used, and methylene chloride when a low vacuum is used, in step Ab).

In step Ab) the solvent may be removed by many conventional means bearing in mind that the sensitivity of the interleukin. Preferred means comprise evaporation under a low vacuum, e.g. 10 to 50 mm Hg, or freeze-drying under a high vacuum, e.g. at below 5 mm Hg, e.g. 0.1 mm Hg.

We have found freeze-drying espeically preferred.

Suitably the step is effected at a temperature below room temperature, and the vacuum and temperature controlled such that the mixture being evaporated is about 1-3 degrees Centigrade cooler than the surroundings. Such control may be effected in conventional manner. A typical program for freeze-drying starts at about −60°C and increases to −15°C over 12 hours. The temperature is then increased to + 10°C and maintained for 2 hours.

In step Ac) the buffer used is preferably a phosphate buffer, e.g. of pH 4-7, e.g. 5-6.5. Preferably the aqueous phase is hypotonic, e.g. less than 300 mosmol/litre, especially less than 290 mosmol/litre. The resultant suspension preferably contains about 0.001 to about 0.2 g lipid per ml.

In step Ad) homogenisation may be provided by ultrasonic radiation. Suitable frequencies of such radiation may be for example from 30 to 80 kHz. A typical power is from 200 to 400 Watts for about 10 ml of mixture being homogenized or agitated. Naturally it is preferred to have the mixture being homogenized separated from any titanium or other metal associated with the ultrasonic radiation generator to avoid contamination by the metal.

The temperature is preferably from about 10 to 70°C. Room temperature is preferred for unsaturated lipids and 60 - 70°C for saturated lipids.

During this stage an emulsion of liposomes with agglomerates may form.

If desired the ultrasonic radiation may be followed by agitation by a high speed mechanical stirrer at e.g. 10,000 to 27,000 rpm. It is preferred, however, to omit this stage, and have agitation provided also by ultrasonic radiation under the same conditions as for homogenization.

In step Ae) the liposomes according to the invention may be isolated according to conventional techniques, e.g. by ultrafiltration, centrifugation, ion-exchange or gel chromatography, dialysis etc. The liposomes may be filtered and sterilized through a filter with suitably small openings, e.g. 0.1 to 1 micron. If desired the filtration may be effected above the phase transition point of the lipid, e.g. from 30 to 70°C. Preferably the liposomes are isolated by high speed centrifugation, e.g. at 10,000 - 20,000 g.

An example of process a) is

a'a) mixing a hydrophilic solution of a interleukin with a lipid in an organic solvent,

a'b) removing the water and organic solvent by evaporation,

a'c) taking up the residue in a buffer solution of a pH 4-7 to form a suspension

a'd) subjecting the suspension to ultrasonic radiation to form liposomes, and

a'e) isolating the liposomes.

Another example of process a) is

a'a) mixing a hydrophilic solution of an interleukin with lipid in an organic solvent,

a'b) freeze-drying the mixture to give a lyophilisate,

a'c) suspending the freeze-drying mixture in a solution of a buffer (pH 4-7),

a'd) subjecting the solution to agitation until liposomes and/or agglomerates are produced,

a'e) homogenization the mixture by mechanical means, and

a'f) isolating the liposomes.

These precesses may be effected in analogous manner to process a).

In another aspect the invention provides a process b) for producing interleukin-containing liposomes which comprises

ba) mixing an aqueous based solution of an interleukin and an organic solution of lipid,

bb) irridating the mixture ultrasonically until some liposomes and/or agglomerates are formed, and

bc) recovering liposomes from the mixture, e.g. by

bi) removing organic solvent from the mixture to form a gel - like intermediate phase, and

bii) converting the gel - like intermediate phase into liposomes.

In step ba) the term "solution" covers "pseudo" solutions like emulsions. However, it is preferred to produce a uniform, clear solution. Any solvent system may be used which will dissolve or solubilize the lipid. The system may be a single solvent or a mixture of solvents. It may contain up to e.g. 15% water. If desired, a survactant may be present. The solvent system may comprise any appropriate organic solvent which can be removed from the lipid by evaporation. A wide variety of ethers, such as diethyl ether, and diisopropyl ether, esters such as ethyl acetate, alcohols such as methanol and tert-butanol, and halogenated hydrocarbons such as methylene chloride and chloroform may be used

If desired acetic acid may be present. It is preferred to use tert-butanol or preferably diethyl ether or methylene chloride.

The interleukin may be dissolved in water or preferably an aqueous buffer system of pH 4-7.4,e.g.5 to 6.5.

Preferably the aqueous buffer is hypotonic, e.g. less than 300 mosmol/litre, especially less than 290 mosmol/litre. The resultant mixture preferably contains about 0.001 to about 0.2 g lipid per ml.

In step bb) irradiation may be provided by ultrasonic radiation. Suitable frequencies of such radiation may be for example from 30 to 80 kHz. A typical power is from 200 to 400 Watts for each 10 ml of mixture being irradiated. Naturally it is preferred to have the mixture being irradiated separated from any titanium or other metal associated with the ultrasonic radiation generator to avoid contamination by the metal.

The temperature is preferably from about 10 to 70°C. Room temperature is preferred for unsaturated lipids and 60-70°C for saturated lipids.

During this stage an emulsion of liposomes with agglomerates may form.

The liposomes may be recovered in conventional manner, and steps taken to optimise their yield.

In step i), the organic solvent is conveniently removed under a low vacuum, e.g. from 10 to 600 mm Hg, whilst still retaining most of the aqueous phase Preferably low temperatures are used, e.g. room temperature or slightly elevated temperatures e.g. to 45°C.

In step ii), the resultant gel like intermediate phase may be treated with water, optionally a buffer solution as described above to produce the liposomes.

In another aspect the invention provides a process c) for producing interleukin-containing liposomes which comprises

ca) mixing a interleukin, a aqueous-based buffer at oH 4-8, and lipid in an organic solvent,

cb) radiating the resultant suspension ultrasonically to form liposomes or agglomerates, and

cc) recovering liposomes from the mixture e.g. by

ci) evaporating the solvent from the mixture, e.g. by a gel - like intermediate phase, and

cii) convering the gel - like intermediate phase into liposomes.

In step ca) the term "solvent" covers "pseudo" solutions like emulsions. However, it is preferred to produce a uniform, clear solution. Any solvent system may be used which will dissolve or solubilize the lipid. The system may be a single solvent or a mixture or solvents. It may contain up to e.g. 15% water. If desired a surfactant may be present. The solvent system may comprise any appropriate organic solvent which can be removed from the lipid by evaporation. A wide variety of ethers, such as diethyl ether, and di-isopropyl ether, esters such as ethyl acetate,alcohols such as methanol and tert-butanol, and halogenated hydrocarbons such as a methylene chloride and chloroform may be used. If desired acetic acid may be present. It is preferred to use diethylether, methylene chloride or tert-butanol.

The interleukin may be used in powder form, e.g. with a particle size of a conveniently maximum particle size of 60 microns in diameter.

In step ca) the buffer used is preferably a phosphate buffer, e.g. of pH 4-7.4, such as 4 to 7, e.g. 5-6.5. Preferably the aqueous phase is hypotonic, e.g. less than 300 mosmol/litre, especially less than 290 mosmol/litre. The resultant suspension preferably contains about 0.001 to about 0.2 g lipid per ml.

In step cb) radiation may be provided by ultrasonic radiation. Suitable frequencies of such radiation may be for example from 30 to 80 kHz. A typical pow r is from 200 to 400 Watts for about 10 ml of mixture being radiated. Naturally it is preferred to have the mixture bein radiated separated from any titanium or other metal associated with th ultrasonic radiation gen rator to avoid contamination by th metal.

The temperature is preferably from about 10 t 70°C. Room temperature is preferred for unsaturated lipids and 60-70°C for saturated lipids.

During this stage an emulsion of liposomes with aggl m rates may form.

The liposomes may be recovered in conventional manner, and steps taken to optimise yields of liposomes. In step ci), the organic solvent is conveniently removed under a low vacuum, e.g. effected by a water pump at for example from 10 to 600 mm Hg, whilst still retaining most of the acueous phase. Preferably low temperatures are used, e.g. room temperatures or slightly elevated temperatures, e.g. to 45°C.

In step cii), the resultant gel - like intermediate phase may be treated with water, optionally a buffer solution as described above to produce the liposomes.

A suspension may form. If desired further evaporation may be effected to remove residue traces of organic solvent and acid liposome formation.

The liposomes according to the invention may be further isolated according to conventional techniques, e.g. as in process step ae).

The purity of the liposomes may be determined by conventional techniques.

To determine the incorporation of interleukin in the liposomes, the prepurification crude mixture may be diluted and centrifuged e.g. for 3 to 24 hours and the amount of interleukin in the supernatant liquor determined. The residueal interleukin is then taken to be incorporated in the liposomal sediment.

Additionally, the incorporation of interleukin in the purified liposomes may be determined by standard high-performance liquid chromatographic techniques as described in the Examples hereinafter.

The distribution of the liposomes according to the invention after administration may also be followed by conventional pharmacokinetic techniques, e.g. in animal tests. Radioactive interleukin is made by reacting iodine-125 with interluekin or by producing the interleukin from amino acids which are radioactively labelled, e.g. with sulphur 35. This interleukin is incorporated into liposomes, which are produced from radioactively labelled lipids, e.g. with carbon-14, and injected into mice. After 1 hour the mice are sacrificed and the amount of and type of radio-activity in each of the organs e.g. spleen, is measured.

The liposomes of the invention may be used in a variety of applications known for interleukins. These applications include use to promote growth of animal cells in culture and other *in vitro* applications and also include therapeutic use in treating a variety of conditions.

In one test hereinafter referred to as the bioassay test a interleukin bioassay is made based on the interleukin concentration dependent stimulation of proliferation of a mouse-T-lymphocyte cell series (CTLL cells) (see S.Gillis et al. J.Immunol. (1978), *120*, p.2027- 2032).

T-cells of a interleukin-dependent cell-culture were washed to remove IL-2 and were treated with liposomes according to the invention, and incubated in an IL- 2 free medium. The test cells were then subjected to a conventional series dilution test (dilution medium RPMI-1640, supplemented with 10% fetal calves serum). The proliferation of the cells after 24 hours incubation measured against increasing liposome concentration. For each dilution 2.10^4 cells were used.

The proliferation ismeasured through the incorporation of [^{3}H]-thymidine or the photometricf MTT test (MTT = (3'(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoline- bromide). The yellow MTT of the living cells in converted into the corresponding dark blue formazan which is measured after dissolution in acidic isopropanol spectro- photochemically.

The dilution of a sample giving 50% of the maximum proliferation rate is measured. The activity of the liposomes according to the invention is about 1.2 to 20 times less than that of pure interleukin. Preferably the liposomes are used which have an activity of greater than 1.3 ng/ml in the above test.

The activity may also be confirmed in standard in vivo tests for IL-2 activity, e.g. in mice with suppressed T-lymphocyte cells e.g. nude mice. In one test such mice are produced by daily administration of 90 mg/kg cyclosporin A whereupon after immunisation with sheep erythrocytes (SRC) the number of specific antibody cells (PFC) in the spleen according to the Jerne Plaque- forming cell assay is reduced by 80 to 97%. After i.v. injection of the antigen the mice are divided up into groups (6-7 animals per group) and given IL-2 preparations in doses containing about 2 to 5 micrograms of IL-2 by sub-cutaneous or preferably i.v. injection twice daily over 5 days. The revival of the immunity of the animals was determined by measuring the number of specific antibody cells in the spleen according to the Jerne test. The activity of the liposomes of the invention is of the same order as the unencapsulated interleukin.

It is preferred to use the liposomes of the invention in therapeutic applications of particular interest based on the recognized immuno-regulatory properties of interleukins, e.g. as mentioned on page 1 of this specification, in particular their use in treatment of conditions due to immunodeficiencies such as the severe combined immuno-deficiency syndrome (SCIDS), the acquired immunodeficiency syndrome (AIDS), immunodeficiency states of old age and congenital immunodeficiency. However, the interleukin may also be used as an anti-myotic agent in the tr atment of various viral diseases such as cytomegalovrius infections and Herpes virus infections and in th treatment of bacterial infections such as Tuberculosis and Leprosis. Dosages required for such therapeutic use of lipos mes will vary depending upon known factors such as the conditi n being treated, th severity of the condition and p tency and amount f the interleukin incorporated, and the like.

However, satisfactory results may b gen rally obtained when administer d to mammals, e.g. human patients, in daily dosages of interleukin ranging from 0.1 μg to 30 μg/kilogram of body weight. Intravenous administration in a suitable sterile vehicle is generally pr ferred.

Among the wide variety of particular uses of interleukin based on its ability to promote T-cell growth in culture are those which find application in diagnostics and in assisting therapeutic treatments. Use in diagnostics is based on the known ability to detect the presence of certain diseases by determining the extent of T-lymphocite growth *in vitro* after addition of the interleukin, using the uptake of radioactive thymidine in the cells as a measure. In assisting therapeutic treatments, such as anti-tumor treatment, the interleukin may be used to promote *in vitro* the growth of T-lymphocytes obtained from the patient or other compatible donor, and the resulting proliferate lymphocytes then reinfused to the patient to assist in combatting the condition. In general, the amount of interleukin to be used to support T-cell growth in culture and other *in vitro* applications may be generally determined from literature accounts of its activity and of its comparison to human interleukin in such ystems, and may be optimized for particular situations by routine investigations.

The following examples illustrate the invention;

As used hereinafter "lecithin" refers to soya-bean lecithin.

Ultrasonic radiation is at 50KHz and 350 W, unless otherwise stated.

PROCESS A

EXAMPLE A1: Preparation of liposomes

2 mg of human IL-2 are dissolved in 1 ml of acetic acid. 1 gram of purified lecithin is separately dissolved in 5 ml tert-butanol. The two solutions are mixed together and freeze dried at −30°C to give a lyophilizate. The lyophilizate is taken up with 0.05 M phosphate buffer (pH 5) to 10 ml giving a suspension.

The suspension is subjected to ultrasonic radiation in a bath for 5 minutes. The suspension then is homogenized for 15 minutes with a high speed stirrer at about 15,000 r.p.m. (Type Polytron Type 10-30) under nitrogen. The liposome solution is then pressed through a sterile filter (0.2 microns) at a temperature greater than the phasetransition point of the lecithin, e.g. 20°C. The resultant liposomal solution is then lyophilised.

If desired the high speed stirrer step may be omitted and the radiation continued for 15 minutes, and/or the liposomes isolated by centrifugation.

ANALYSIS OF LIPOSOMES

The liposomes of the invention may be analysed using conventional reversed phase high performance liquid chromatographic (HPLC) techniques.

It is preferred to use wide pore (ca 300 angstroms) spherical C-8 hydrocarbon support material, 10 microns (e.g. column RP 300 Aquapore 20 × 4.6 mm) available from Browlee Laboratories Inc. USA).

A preferred eluant system is a water/acetonitrile gradient with 0.1% trifluoroacetic acid, e.g. using two eluant systems.

System A: 50% acetonitrile in H_2O (+ 0.1% trifluoroacetic acid)

System B: 70% acetonitrile in H_2O (+ 0.1% trifluoroacetic acid).

Typical flow rates are 3 ml/minutes and with a gradient of system 100 percent A to 100 percent system B over 10 minutes. Under such preferred conditions human IL- 2 is eluted with a retention time of about 7.5 minutes and the amount of interleukin can be obtained by integration of the peak in conventional manner.

The liposomes of the invention are pre-treated before HPLC

i) by dilution with acetate buffer of pH 2.5 to 4 after which the liposome material breaks down on the HPLC column releasing the interleukin,

ii) by exhaustive removal of liposome excipient from the interleukin incorporated in the liposomes, e.g. water and methanol/methylene chloride to remove phospholipids and then injection of the resultant aqueous solution containing interleukin,

iii) by column switching technique where the liposomes excipients from the interleukin incorporated in the liposomes of the invention are separated on a cation ion exchanger. Afterwards interleukin is eluted from the cation ion exchanger to a reversed phase column where it is chromatographed in the usual way. A suitable cation ion exchanger column has 30 × 4.6 mm in size, packed with 10 µm support (f.e. SC × 10 available from Browlee Laboratories Inc. USA). The liposomes are found to incorporate about 50% of the human IL-2.

ACTIVITY AND UTILITY

The liposomes are found to show the same ord r of activity as human IL-2 itself in the above-mentioned bioassay test.

EXAMPLE A2: Preparation of lyposomes (V3)

0.5 mg human IL-2 are dissolved in 1 ml acetic acid. 500 mg of lecithin (EPIKURON 145 V) is separately dissolved in 50 ml t-butanol. The two solutions are mixed together and freeze dried at −15°C over about 16 hours to give a lyophilizate. The lyophilizate is taken up with 0.05 M phosphate buffer (pH 6.5) to 10 ml giving a suspension.

The suspension is subjected to ultrasonic radiation in a bath for 5 minutes at room temperature (ca 20°C). The suspension is then homogenized for 10 minutes with a high speed stirrer at about 15.000 r.p.m. (Type Polytron PT 15) at room temperature.

The liposomes are isolated by centrifugation at 150,000 g at 4°C for 4 hours.

EXAMPLE A3: Preparation of liposomes

Examples A2 is repeated with ultrasonic radiation and high speed stirrer homogenization at 70°C instead of room temperature. The lecithin used was Soja phosphatid NC 95H. Centrifugation time was 2 hours.

EXAMPLE A4: Preparation of liposomes

0.5 mg of human IL-2, 450 mg lecithin (Epikuron 145 V) and 50 mg phosphatidylserine are mixed together in 10 ml tert-butanol and irradiated for 10 minutes at room temperature to form a solution. The solution is freeze-dried at −40°C over about 18 hours to form a lyophilisate.

The lyophilisate is taken up with 0.05 M phosphate buffer (pH 6.3) to 10 ml giving a suspension. The suspension is subjected to ultrasonic irradiation in a bath for 10 minutes at room temperature (20°C) and then homogenised as in Example 2 for 10 minutes. The liposomes are isolated by centrifugation at 150,000 g at 4°C for 4 hours.

EXAMPLE A5: Preparation of liposomes

Example A4 is repeated using 500 mg of lecithin in place of 450 mg lecithin and 50 mg phosphatidylserine.

EXAMPLE A6: Preparation of liposomes

Example A4 is repeated using 425 lecithin and 75 mg cholesterin in place of 450 mg lecithin and 50 mg phosphatidylserine.

EXAMPLE A7: Preparation of liposomes

Example A2 is repeated without the high speed stirring step. Ultrasonic irradiation continued for 15 minutes.

EXAMPLE A8: Preparation of liposomes

Examples A 3-6 are each repeated without the high speed stirring step.

ANALYSIS OF LIPOSOMES

Supernantant liquid

The supernatant liquid after centrifugation is analysed by reversed phase high performance liquid chromatographic techniques as described above and contain less than 10% of the original human IL-2.

Biological activity

The liposomes are analysed according to the series dilution test on the incorporation of ^{3}[H]-thymidine in IL-2 dependent CTLL-(16) cells in the test described above (S.Gilles et al). The concentration of IL-2 which produces 50% of the maximal growth of 10^4 cells after 24 hours is measured immediately after preparation (Value A in µg/ml). In this test the substance human IL-2 itself used as starting material for liposome formation had a value of 0.2 µg/ml.

Results

Example	A^0 (ng/ml)	A^2 (ng/ml)	A^4 (ng/ml)
A2	0.2	0.7	3.2
A3	9.5	8.4	nt
A4	0.7	nt	nt
A5	2.6	nt	nt
A6	1.6	nt	nt
A7**	1.0	nt	nt

* storage after 4 w eks at 4°C

** initial IL-2 charg 0.8 ng/ml activity

nt = not tested

In the abov vivo test the following results were obtained with a stored batch of the Example A2 liposomes having an in vitro activity in the ab ve test of 1.7 ng/ml:

	Dose in micro grams	PFC in spleen	Activity relative to activity of control
Immunized non-suppressed mouse (Control)	-	167,000	100%
Suppressed mouse	-	28,000	17%
IL-2 substance	5	111,000	66%
Liposomes	5	96,700	58%
IL-2 substance	2	96,700	58%
Liposomes	2	87,100	52%

EXAMPLE A9: Preparation of liposomes

0.5 mg human IL-2 is dissolved in 1 ml acetic acid and the solution is added to a solution of 500 mg lecithin (Epikuron 145 V) in 50 ml methylene chloride.

The mixture is evaporated in a rotary evaporator at 30°C under a vacuum of 10 to 50 mm Hg for 1 hour and then at 50°C for 3 hours to give a lipid film. The film is dispersed in 10 ml (0.02 M) phosphate buffer at pH 5. The dispersion is then irradiated in an ultrasonic bath for 30 minutes at room temperature (20°C) to form liposomes.

The liposomes are isolated by centrifugation at 15,000 g at 40°C for 4 hours.

EXAMPLE A10: Preparation of liposomes

Example 9 is repeated with ultrasonic irradiation at 70°C instead of room temperature. The lecithin used was Soja phosphatic NC 95 H. The centrifugation is effected for 2 hours.

ANALYSIS OF LIPOSOMES

Analogues to analysis effected for EXAMPLE A4

Results

Example	A^0 (ng/ml)	A^2 (ng/ml)
A9	0.8	0.9
A10	2.5	3.5

PROCESS B

EXAMPLE B1: Preparation of liposomes

100 mg of lecithin are dissolved in 5 ml methylene chloride and placed in a round bottomed flask. An aqueous solution of 0.2 mg human IL-2 and 20 mg L-Cysteine in 1 ml 0.05 M phosphate buffer (pH 5) is added. The mixture is emulsified in an ultrasonic bath for 5 minutes at 20°C (80 kHz frequency).

The emulsion (containing some preformed liposomes and agglomerates) is then concentrated at 10°C in a vacuum (20 to 30 mm Hg) to give a gel to which is added 2 ml of the buffer solution whilst the mixture is slowly stirred. An aqueous liposomal suspension results.

The liposomes are separated from the non-incorporated active agent by the following steps:-

a) centrifugation (5 minutes, 20°, 5000 G). The non- incorporated active agent remains in the supernatant liquid and the liposome sediment is resuspended.

b) gel column chromatography as (Biogel A 1.5 or Sephadex G 50). The liposome-containing eluate is concentrated by ultrafiltration or dialysis against a 0.9% brine solution.

The resultant liposomal suspension is lyophilisated.

ANALYSIS

The liposomes are found to incorporate about 50-90% of the human IL-2.

ACTIVTY AND UTILITY

The liposomes are found to show the same order of activity as human IL-2 itself in the above-mentioned bioassay test.

EXAMPLE B2: Preparation of liposomes

500 mg of lecithin (Epikuron 145V) are dissolved in 200 ml methylene chloride and placed in a round bottomed flask. An aqueous s lution of 0.5 mg human IL-2 and 100 mg L-Cystein in 10 ml 0.05 M phosphat buffer (pH 5) is added.

The mixture is emulsified in an ultrasonic bath for 10 minutes at toom temperature (20°C). Th result-ant emulsion (containing some preformed liposomes and agglom rates) is then concentrated at 35°C for 10 minut s in a vacuum (20 to 30 mm Hg) to giv a g l to which 10 ml of buff r solution is added. The mixture is stirred for 30 minutes at room temperature.

The liposomes are isolated by centrifugation at 150,000 g at 4°C for 4 hours.

EXAMPLE B3: Preparation of liposomes

Carried out in analogous manner to Example B2, but concentrating at 80°C for 5 minutes instead of at 35°C for 10 minutes. The lecithin used was Soja phosphate NB 95 H. The centrifugation was effected for 2 hours instead of 4 hours.

EXAMPLES B4 and B5: Preparation of Liposomes

Carried out in analogous manner to examples 2 and 3, but without L-Cysteine being present.

ANALYSIS of Liposomes

Supernatant Liquid

The supernatant liquid after centrifugation is analysed by reversed phase high performance liquid chromatographic techniques as described above and contain less than 10% of the original human IL-2.

Biological activity

The liposomes are analysed according to the series dilution test on the incorporation of ^{3}[H]-thyridine in IL-2 dependent CHL-(16) cells in the test described above (S.Gilles et al). The concentration of IL-2 which produces 50% of the maximal growth of 10^4 cells after 24 hours is measured immediately after preparation (Value A° in ng/ml) and after 2 weeks storage at 4°C (Value A^2 in ng/ml). In this test the substance human IL-2 itself had a value of 0.2 ng/ml.

Results

Example	A° (ng/ml)	A^2 (ng/ml)
B2	0.8	2.4
B3	20.0	35.0

PROCESS C

EXAMPLE C1: Preparation of Liposomes

50 micromoles of purified lecithin and 50 micromole of cholesterol are dissolved in 20 ml chloroform and evaporated in a rotary evaporator under nitrogen. The residue is taken up in 5 ml diethyl ether. 600 micrograms human Il-2 (as a powder e.g. particle size 5 to 50 microns) are added. To this solution 1.5 ml of 0.05 molar phosphate buffer are added.

The mixture is then irradiated with an ultrasonic probe at 5°C for 5 minutes to give a homogenisate. The resultant emulsion is concentrated in a rotary evaporator in two stages. In the first stage the pressure is about 400 Torr Hg and maintained until there is a viscous gel. This gel is treated with 1.5 ml 0.05 M phosphate buffer (pH 5), and the container shaken slightly to give an aqueous suspension. The aqueous suspension is then subjected to a second evaporation stage at RT and normal pressure for 15 minutes to give a non-transparent suspension of liposomes.

To remove the last traces of organic solvent an ultra- filtration over an ion-exchange column (Sephadex 650) may be carried out.

ANALYSIS of Liposomes

The liposomes are found to incorporate about 50-90% of the human IL-2.

ACTIVITY and UTILITY

The liposomes are found to show the same order of activity as human IL-2 itself in the above-mentioned bioassay test.

EXAMPLE C2: Preparation of Liposomes

500 mg of lecithin (EPIKURON 145 V) and 0.5 mg human IL-2 are dissolved in 20 ml methylene chloride. 10 ml 0.05 molar phosphate buffer is added.

The mixture is then irradiated in an ultrasonic bath for 5 minutes at room temperature (ca 20°C). The resultant emulsion is concentrated in a rotary evaporator at 35°C for 10 minutes, until there is a viscous gel. 10 ml 0.05 M phosphate buffer solution (pH 5) is then added and the container rotated for 30 minutes at room temperature.

The liposomes are isolated by centrifugation at 150,000 g at 4°C for 4 hours.

EXAMPLE C3: Preparation of Liposomes

Example C4 is repeat d with c ncentration at 80° for 5 minutes Instead f 35°C for 10 minuts. The lecithin was Soja phosphatic NC 95 H. Th centrifugati n was effected for 2 hours.

ANALYSIS of Liposomes

Supernatant liquid

The supernatant liquid after centrifugation is analysed by reversed phase high performance liquid chromatography t chniqu s as described abov and contain less than 10% of the original human IL-2.

Biological activity

The liposomes are analysed according to the series dilution tests on the incorporation of ^{3}[H]-thyridine in IL-2 dependent CHLL-(16) cells in the test described above (S.Gilles et al).

The concentration of IL-2 which produces 50% of the maximal growth of 10^4 cells after 24 hours is measured immediately after preparation (Value A^0 in ng/ml) and after 2 weeks storage at 4°C (Value A^2 in ng/ml). In this test the substance human IL-2 itself had a value of 0.2 ng/ml.

Results

Example	A^0 (ng/ml)	A^2 (ng/ml)
C2	1.2	2.9
C3	12.5	20.0

In any of the above Examples of processes A, B or C the human IL-2 may be replaced by IL-2-serine 125, IL-2 A-1, IL-2 A-2, IL-2 B-1, IL-2 B-2, IL-2 Gln 26, IL-2 Phe-121 or IL-2 Stop 121.

CLAIMS

1. Interleukin- containing liposomes.
2. Liposomes according to claim 1 wherein the interluekin is human IL-2.
3. Liposomes according to claim 1 wherein the interluekin is IL-2-serine 125.
4. Liposomes according to claim 1 wherein the interleukin is IL-2 A-1, IL-2 A-2, IL-2 B-1 or IL-2 B-2.
5. Liposomes according to claim 1 wherein the interleukin is IL-2 Gln-26, IL-2 Phe-121 or IL-2 Stop-121.
6. An improved method of administering a interleukin to a subject in need of interleukin treatment which comprises intravenously administering a interleukin containing liposome of any one of claims 1 to 5.
7. A method for a solution of an interluekin with lipid in an organic solvent,
a) forming a solution of an interleukin with lipid in an organic solvent,
b) removing the solvent from the solution to give a residue,
c) suspending the residue in a solution of a buffer,
d) subjecting the suspension to agitation and homogenization until liposomes are produced, and
e) isolating the liposomes.
8. A method for producing interleukin containing liposomes which comprises
a) mixing an aqueous solution of an interleukin and an organic solution of a lipid,
b) subjecting the mixture to ultrasonic radiation until some liposomes and/or agglomerates are formed, and
c) recovering liposomes from the mixture.
9. A method for producing interleukin containing liposomes which comprises
a) mixing a interleukin, an aqueous - based buffer at pH 4-8, and lipid in an organic solvent,
b) radiating the resultant suspension ultrasonically to form some liposomes or agglomerates, and
c) recovering liposomes from the mixture.
10. A method according to claim 9 wherein the buffer has a pH of 4 to 7.
11. A method according to claim 9 wherein the liposomes are recovered by
i) evaporating the solvent from the mixture to form a gel-like intermediate phase, and
ii) converting the gel-like intermediate phase into liposomes.
12. A method according to claim 8 wherein the liposomes are recovered by
i) evaporating the solvent from the mixture to form a gel-like intermediate phase, and
ii) converting the gel-like intermediate phase into liposomes.
13. A method for producing interleukin containing liposomes which comprises
a) mixing a hydrophilic solution of a interleukin with a lipid in an organic solvent,
b) removing the water and organic solvent by evaporation,
c) taking up the residue in a buffer solution of a pH 4-7 to form a suspension,
d) subjecting the suspension to ultrasonic radiation to form liposomes, and
e) isolating the liposomes.
14. A method for producing interleukin containing liposomes which comprises
a) mixing a hydrophilic s lution of an int rleukin with lipid in an rganic s lvent,
b) freeze-drying the mixture to give a lyophilisate,
c) suspending the freeze-dried mixtur in a soluti n of a buffer (pH 4-7),
d) subjecting th solution to agitation until liposomes and/or agglomerates ar produced,
e) homogenizing the mixture by m chanical means, and
f) isolating th liposomes.

15. A method for producing interleukin containing liposomes substantially as hereinbefore described with respect to any one of the Examples.

16. Liposomes whenever produced by a process according to any one of claims 6 to 15.

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